



*Considered  
Dm 9/3/04*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner: Richard A. Schnizer

Serial No.: 09/328,975 )

Filed: 06/09/1999 )

Group Art Unit: 1635 )

For: Charge Reversal of Polyion Complexes

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.131

Dear Sir:

I, an inventor, Vladimir S. Trubetskoy, hereby declare as follows:

1. I am an inventor of the captioned application.
2. Photocopies of pages from my, Vladimir Trubetskoy's, personal laboratory notebook showing recharging of DNA/polycation particles beginning on December 16, 1997 accompany this Declaration.
3. It is known to me that the process performed in the notebook pages results in the formation of negatively charged tertiary complexes as described in the present specification.
4. The recharging process was conceived prior to the effective date of the Office Action prior art reference.
5. Developed of the recharging process occurred with due diligence from conception to the filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

  
Vladimir S. Trubetskoy      Date

*6/7/04*



450 65  
100 10

(1)

product

1.5 ml of react mix applied to  
Krich preparative TLC silica  
plate and run in  
 $\text{CHCl}_3/\text{MeOH}$  (65:20) system.

Product band was scraped off the  
plate.

12/16/97

Work with BTDNA-lis MS

Part of silica (above) was washed with

(1)  $\text{CHCl}_3$

(2)  $\text{CHCl}_3/\text{MeOH}$  65:20

Substantial amounts of  
BSS (upper spot is present)

Whole amount of silica was washed with

(1)  $\text{CHCl}_3/\text{MeOH}$  65:10

(2) 6:11 — 65:30 → this fraction  
was evaporated

Work on recharging surface of caged DNA particles.

Caged particles are positively charged. If you add  
excess of polyanion it can recharge the surface  
to the opposite charge.

Caged particles were prepared in Bulker's  
conditions (p. 72)

After 2h of incubation of react mix at room to

The mixture was diluted twice with deionized  $H_2O$

and to 12% DNA/48% PLL caged, 500% of polymethacrylic acid (pMAA) were added.

No.	FI	Conc.
1	239.385	-10408 DNA/PLL (1:6) capped 1.7 DTBP
2	525.217	-22835 +500% pMAA
3	392.396	-17060 after centrifug.
4	720.091	-31308 +150mM NaCl
5	481.248	-20923 after centrifug.

$\zeta$ -potential was also measured

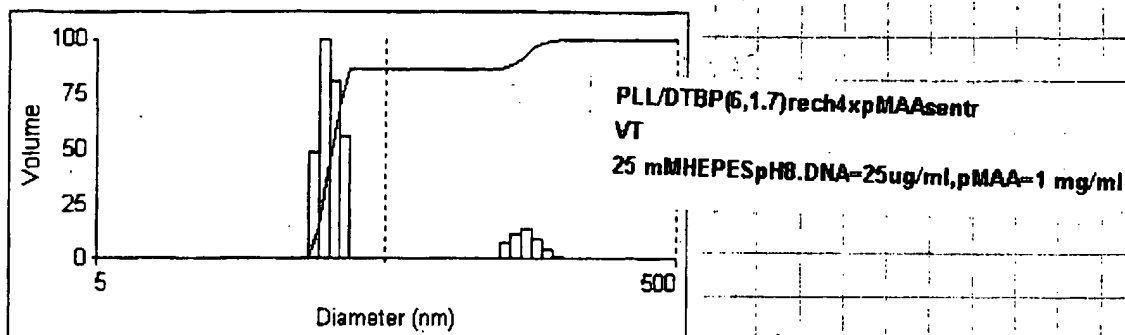
Run	Zeta Potential (mV)	Half Width (mV)
1	7.66	2.34
2	8.01	2.18
3	8.08	2.22
4	10.20	2.58
5	8.06	2.63
6	6.74	2.25
7	6.69	2.29
8	23.20	2.26
9	8.05	2.24
10	27.46	4.86
Mean	11.41	2.58
Std. Error	2.36	0.26

PLL/DTBP(6,1.7)nosalt (Run 10)  
VT  
DNA=17ug/ml, 17 mM HEPES, pH 8.0

Run	Zeta Potential (mV)	Half Width (mV)
1	-29.03	2.80
2	-7.70	4.06
3	-16.37	2.74
4	-25.43	3.63
5	-63.89	2.89
6	-16.63	2.89
7	-28.26	2.63
8	-24.13	3.00
9	-26.00	7.24
10	-36.16	4.16
Mean	-26.15	3.59
Std. Error	3.97	0.44

PLL/DTBP(6,1.7)+4xpMAAnosalt (Run 10)  
VT  
DNA=17ug/ml, 17 mM HEPES, pH 8.0

After addition of pMAA,  $I_{50}$  is increasing somewhat but still particle sizing.



30/24/1142/152

Basically the same effect was observed with dextran-sulfate(DS) as counterion.

The mixture was as indicated on p 75 with exception that DS was added ~~as~~ instead of pMAA

Run	Zeta Potential (mV)	Half Width (mV)
1	33.22	2.41
2	27.98	2.61
3	20.17	3.26
4	26.99	2.22
5	10.37	2.36
6	27.01	2.06
7	33.33	2.24
8	26.83	4.46
9	28.83	2.93
10	<del>29.38</del>	2.18
Mean	26.31	2.67
Std. Error	2.13	0.23

PLL/DTBP(6,1.7)nosalt (Run 10)  
VT  
DNA=25ug/ml, 25 mM HEPES, pH 8.0, DS=0.5mg/ml

Run	Zeta Potential (mV)	Half Width (mV)
1	-7.34	2.32
2	-22.67	2.92
3	-13.63	2.19
4	-16.96	6.66
5	-2.66	3.97
6	-21.18	2.28
7	-26.78	2.10
8	-13.92	2.42
9	-11.06	2.01
10	-16.94	6.32
Mean	-15.00	3.21
Std. Error	2.23	0.60

PLL/DTBP(6,1.7)+500ugDSnosalt (Run 10)  
VT  
DNA=25ug/ml, 25 mM HEPES, pH 8.0, DS=0.5mg/ml

25 mg P-2538 Lot 75H5551

**SIGMA**  
POLY-L-LYSINE  
Hydrobromide

CAUTION: The chemical, physical and toxicological properties of this product have not been thoroughly investigated. Exercise due care.

Desiccate

Store at less than 0°C

DP(vis) 251  
MW(vis) 52,400  
DP(LALLS) 252  
MW(LALLS) 52,700  
Mw(MSEC-LALLS) 1.10

For laboratory use only. Not for household or other uses.  
NDS available

SIGMA CHEMICAL CO. P.O. Box 14008 St. Louis, MO 63118 USA Tel: 314-771-5100

12/17/97 Titrations of DNA/PLL (1:6) caged and non-caged with dextran sulfate.

Buckler's solution was prepared as described in p. 72 this volume

V = 1.5 ml (30 g DNA / 114 g PLL)

50 g - 500 g of dextran sulfate ( $M_w = 500$  kDa, Sigma) were added to each sample and

$I_{50}$ ,  $F_{0.0}$ , size and  $\zeta$ -potential were measured. Some non-caged samples were measured in the same conditions

TOTO concentrations: (8% of stock TOTO into 20 ml of 25 mM HEPES, pH 8.0; 10% of sample  $\rightarrow$  0.5 ml TOTO)

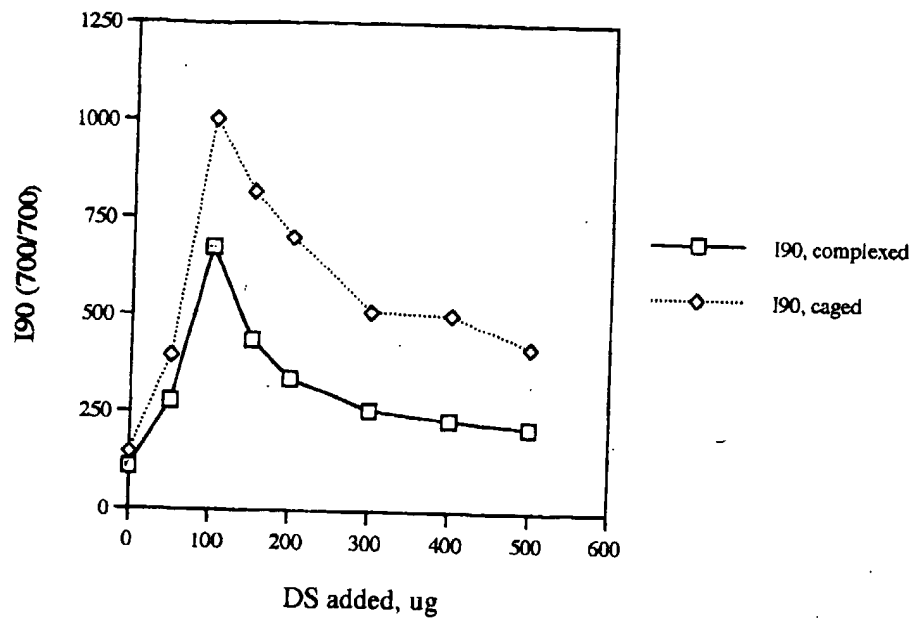
No. Caged	FI	Conc. $I_{50}$ 200/200
1	145.344	-6319.3 0
2	395.046	-17175 50%
3	1004.619	-43679 100%
4	819.067	-35611 150
5	702.273	-30533 200
6	512.809	-22296 300
7	504.484	-21934 400
8	421.555	-18328 500%

No.	FI	Conc. TOTO
1	28.999	-1260.8 $F_0$
2	687.116	-29874 $F_{max}$ 659
3	47.693	-2073.6 0
4	38.309	-1665.6 50
5	72.144	-3136.7 100
6	234.264	-10185 150
7	203.611	-8852.7 200
8	175.301	-7621.8 300
9	161.145	-7006.3 400
10	160.371	-6972.7 500

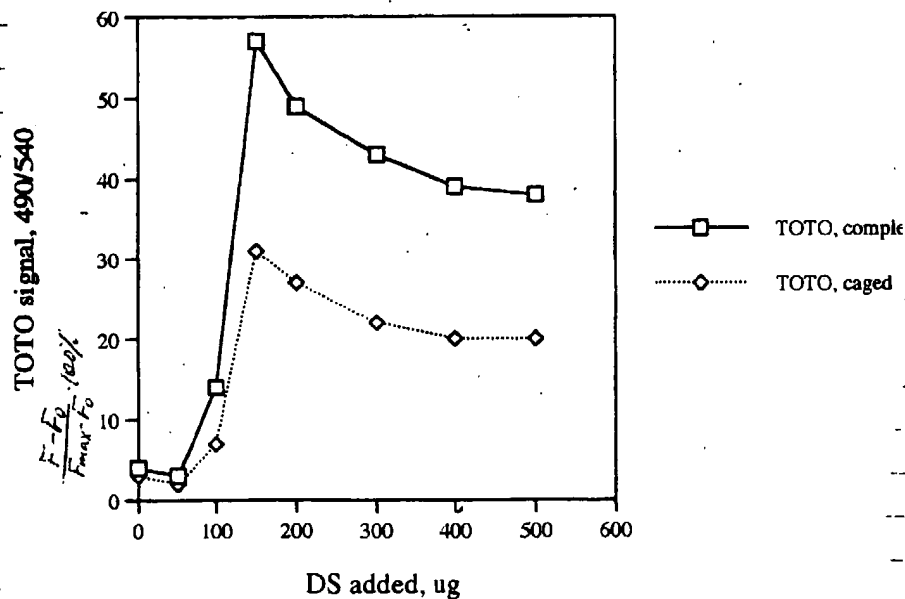
No.	FI	Conc. Complexed
1	108.628	-4723.0
2	278.651	-12115
3	676.371	-29407
4	435.570	-18937
5	338.690	-14725
6	258.092	-11221
7	234.890	-10212
8	215.716	-9379.0

No.	FI	Conc.
1	96.057	-4170.4
2	533.456	-23193 $F_{max}$ 480
3	64.342	-2797.5 0
4	60.599	-2634.7 50
5	111.724	-4857.6 100
6	322.742	-14032 150
7	284.332	-12362 200
8	253.480	-11020 300
9	236.314	-10274 400
10	230.641	-10027 500
11	43.587	-1895.1 $F_0$

### Stabilization of DNA/PLL complexes (caged and complexed) with dextran sulfate



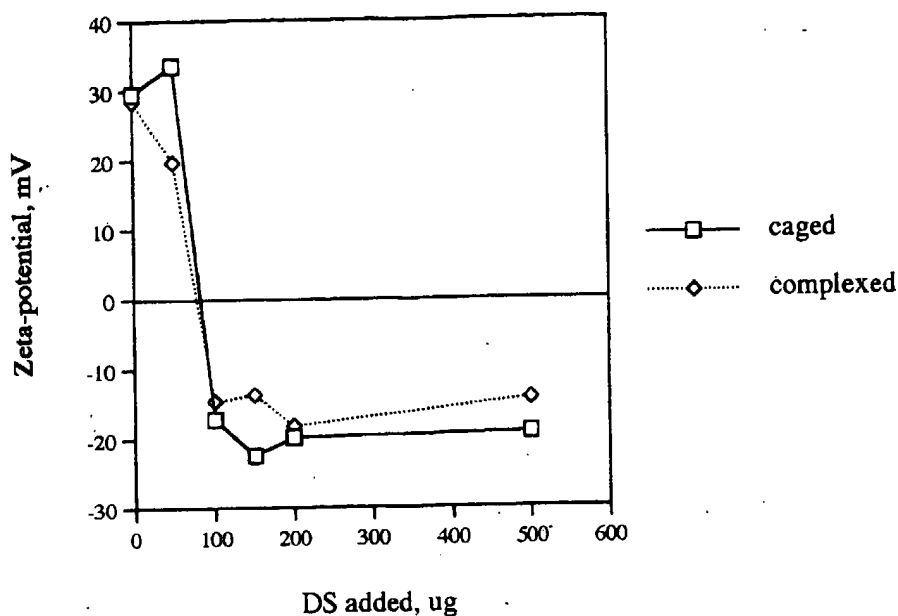
### Condensation of DNA/PLL/DS complexes (caged and complexed)



Complexed DNA/PLL were prepared in the same conditions as for caged but w/o  $\gamma$ -linking with DTBP.

$\zeta$ -potential is changed to opposite at 100  $\mu$ g DS added.

## Zeta-potential of DNA/PLL/DS complexes , no salt



Run	Zeta Potential (mV)	Half Width (mV)
1	31.34	3.67
2	33.02	2.11
3	26.96	3.57
4	39.37	1.96
5	30.17	2.31
6	24.26	2.10
7	26.53	1.95
8	22.45	2.10
9	29.20	1.86
10	29.55	2.96
Mean	29.28	2.48
Std. Error	1.51	0.22

PLL/DTBP(6,1.7) (Run 10)  
VT  
DNA=20ug/ml, 25 mM HEPES, pH 8.0

Run	Zeta Potential (mV)	Half Width (mV)
1	49.36	3.06
2	44.33	1.83
3	37.00	1.80
4	33.83	3.36
5	39.11	2.34
6	27.81	1.81
7	28.87	4.53
8	11.79	1.82
9	36.92	1.84
10	28.00	3.19
Mean	33.68	2.58
Std. Error	3.30	0.30

PLL/DTBP(6,1.7)+50ugDS (Run 10)  
VT  
DNA=20ug/ml, 25 mM HEPES, pH 8.0

Run	Zeta Potential (mV)	Half Width (mV)
1	-18.29	1.86
2	-8.36	1.87
3	-6.31	1.93
4	-14.62	1.93
5	-14.66	1.89
6	-21.63	1.83
7	-18.70	1.81
8	-25.87	2.50
9	-22.83	2.46
10	-21.59	2.07
Mean	-17.26	2.01
Std. Error	1.99	0.08

PLL/DTBP(6,1.7)+100ugDS (Run 10)  
VT  
DNA=20ug/ml, 25 mM HEPES, pH 8.0

4	39.37	1.96
5	30.17	2.31
6	24.26	2.10
7	26.53	1.96
8	22.46	2.10
9	29.20	1.86
10	29.55	2.96

PLL/DTBP(6,1.7) (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	29.28	2.46
Std. Error	1.54	0.22

Run	Zeta Potential (mV)	Half Width (mV)
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1	49.36	3.06
2	44.33	1.83
3	37.00	1.80
4	33.83	3.36
5	39.11	2.34
6	27.81	1.81
7	28.67	4.53
8	11.79	1.82
9	36.92	1.84
10	28.00	3.19

PLL/DTBP(6,1.7)+50ugDS (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	33.68	2.66
Std. Error	3.30	0.30

Run	Zeta Potential (mV)	Half Width (mV)
-----	---------------------	-----------------

1	-18.29	1.86
2	-8.36	1.87
3	-6.31	1.93
4	-14.62	1.93
5	-14.56	1.89
6	-21.63	1.83
7	-18.70	1.81
8	-25.67	2.50
9	-22.83	2.46
10	-21.59	2.07

PLL/DTBP(6,1.7)+100ugDS (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	-17.26	2.01
Std. Error	1.99	0.08

Run	Zeta Potential (mV)	Half Width (mV)
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1	-19.49	1.61
2	-30.43	3.32
3	-21.66	1.68
4	-20.73	1.63
5	-19.74	1.83
6	-21.84	3.94
7	-20.72	1.70
8	-30.38	2.06
9	-16.76	2.26
10	-22.71	1.92

PLL/DTBP(6,1.7)+150ugDS (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	-22.46	2.20
Std. Error	1.42	0.26

Run	Zeta Potential (mV)	Half Width (mV)
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1	-19.39	3.39
2	-23.80	2.03
3	-15.61	1.90
4	-19.76	2.17
5	-17.92	2.76
6	-17.77	1.71
7	-22.13	4.28
8	-26.06	3.88
9	-18.99	1.92
10	-17.96	1.99

PLL/DTBP(6,1.7)+200ugDS (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	-19.84	2.60
Std. Error	0.93	0.29

Run	Zeta Potential (mV)	Half Width (mV)
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1	-17.23	2.37
2	-8.34	1.96
3	-13.48	4.20
4	-23.76	1.84
5	-18.77	1.89
6	-16.69	4.34
7	-23.00	1.95
8	-23.10	2.04
9	-22.88	2.12
10	-26.96	1.84

PLL/DTBP(6,1.7)+500ugDS (Run 10)

VT

,DNA=20ug/ml, 25 mM HEPES, pH 8.0

Mean	-19.21	2.46
Std. Error	1.76	0.31

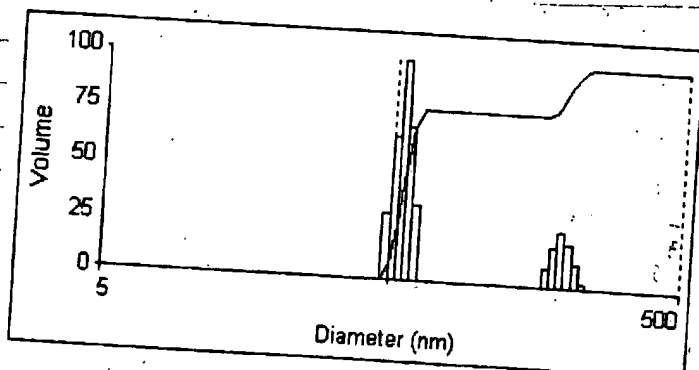
12/18/97

Work on recharged ~~cellulose~~ DNA colloid  
(precipitation in salt)

Samples prepared 12/12 (p27) were tested on precipitation  
upon addition of NaCl up to 150 mM.  
Was done with one sample 150  $\mu$ g DS (close to neutrality point)

No.	$I_{50}$ (700/700) FI	Conc.	
1	396.680	-17246	coupl 150 $\mu$ g DS
2	771.010	-33522	caged - " -
3	356.424	-15496	alt cut - " -
4	484.668	-21072	alt cut - " -
5	640.237	-27836	+ salt
6	667.412	-29017	+ salt
7	<del>618.884</del>	<del>26012</del>	
8	<del>681.895</del>	<del>29621</del>	
9	360.949	-15693	alt cut
10	400.766	-17424	alt cut

There is some  
aggregates formed  
after each step  
but significant  
amounts of particles  
stays in solution  
after addition of salt  
and centrifugation



Caged sample  
produced  
significant intensity  
(1.2 Mops) after  
centrifugation

DNA/PLL (1:6) caged stabilized w 150  $\mu$ g DS  
in 150 mM NaCl after centrifugation

12/23/97

Recharging the colloid with pMAA (not caged)

In standard settings. Complexes were formed  
at DNA 50  $\mu$ g/ml in 25 mM HEPES pH 8. PLL/DNA = 6:1,  $V = 0.5$  ml (25  $\mu$ g/55  $\mu$ l)  
→ then pMAA was added  
thru each 0.5 ml was diluted to 1.5 ml with  
the same buffer  
Igo, TOTO and  $\zeta$  potential were measured

0 - 500  $\mu$  pMAA was added to each 25  $\mu$  DNA sample

No.	FI	Conc.
11	169.143	-7354.0
No.	FI	Conc.

1	177.670	-7724.8
2	695.225	-30227
3	995.999	-43304
4	320.682	-13942
5	603.757	-26250
6	316.927	-13779
7	456.850	-19863
8	305.441	-13280

No.	FI	Conc.
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1	735.620	-31983
2	68.286	-2969.0
3	64.389	-2799.5
4	580.993	-25260
5	708.999	-30826
6	698.460	-30367
7	744.805	-32382
8	741.753	-32250
9	766.905	-33343
10	45.911	-1996.1

No.	FI	Conc.
-----	----	-------

1	125.019	-5435.6
2	339.144	-14745
3	1001.452	-43541
4	964.944	-41954
5	644.407	-28017
6	634.971	-27607

No.	FI	Conc.
-----	----	-------

1	393.592	-17112
2	54.019	-2348.7
3	45.936	-1997.2
4	47.624	-2070.6
5	359.647	-15636
6	225.945	-9823.7
7	206.946	-8997.7

32 - F<sub>0</sub>

pMAA  
Iso (500/600)

pMAA  
mtDNA  
690 0  
23 0  
19 25  
535 50  
663 100  
653 200  
699 300  
696 400  
721 500

F<sub>0</sub>  
25  
Iso

DNA  
355  
0 16  
25 7  
50 9  
100 321  
200 187  
300 168 47%

12/30/97

Recharging the DNA/PLL colloid (uncaged)

repetition of experiments from previous page

## TOTO

No.	FI	Conc.	PHAA	F <sub>0</sub>	F <sub>V</sub>
1	13.291	-577.87		881	100
2	894.401	-38886		81	92
3	94.502	-4108.8		326	37.0
4	339.541	-14762		831	94.3
5	844.788	-36729		888	100.7
6	901.778	-39207		948	104.2
7	931.606	-40504		948	107.6
8	961.974	-41824		965	109.5
9	978.774	-42555			

No.	FI	Conc.	PHAA - <del>DS</del>
1	14.718	-639.91	
2	12.247	-532.48	25
3	11.329	-492.57	50
4	12.886	-560.26	100
5	12.353	-537.09	200
6	12.194	-530.17	300
7	12.591	-547.43	500

No.	FI	Conc.	DS	F <sub>0</sub>	F <sub>V</sub>
1	29.793	-1295.3		856	100
2	868.746	-37771		74	8.6
3	86.448	-3758.6		50	5.8
4	62.691	-2725.7		146	17.0
5	158.887	-6908.1		842	98.4
6	854.383	-37147		421	49.2
7	433.794	-18860		359	41.9
8	371.326	-16144		333	38.9
9	345.736	-15032			

No.	FI	Conc.	DS - DNA
1	15.943	-693.17	
2	12.170	-529.13	25
3	11.950	-519.57	50
4	12.479	-542.57	100
5	12.135	-527.61	200
6	14.364	-624.52	300
7	12.913	-561.43	500

Conditions are the same as in p. 80.

TOTO signals from

PHAA alone and

DS alone were measured.

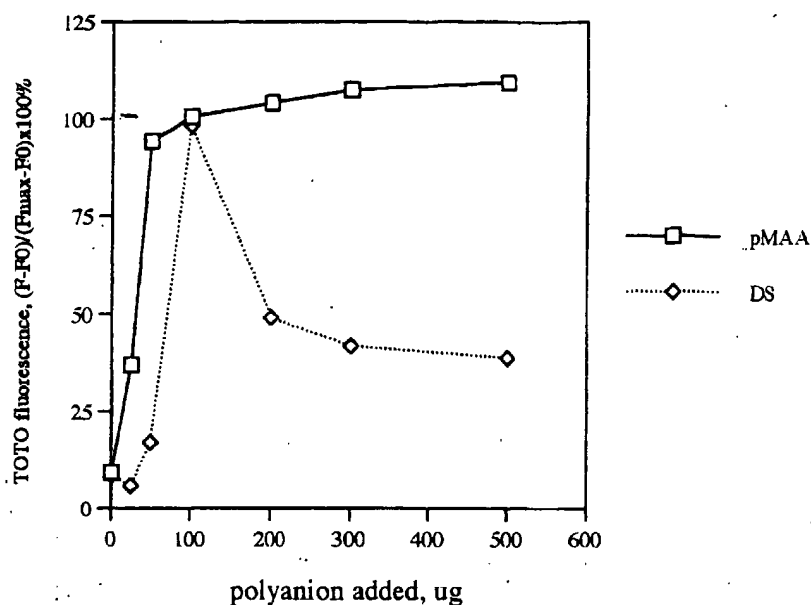
polyanions did not change TOTO signals from DNA.

## Iso (100/600)

No.	FI	Conc.	Z-potential	DS
1	40.148	-6093.4	0	
2	327.189	-14225	25	
3	1008.335	-43840	50	
4	753.784	-32773	100	
5	559.717	-24335		
6	408.500	-17760		
7	332.728	-14466		

No.	FI	Conc.	PHAA
1	337.505	-14674	+
2	1008.335	-43840	+
3	1008.335	-43840	-
4	503.257	-21880	-
5	203.894	-8865.0	100
6	177.915	-7735.4	-
7	135.729	-5901.3	-

Condensation of DNA/PLL(1:6) upon addition of polyanions



1/6/98

Precipitation of DNA/PLL complex after recharging with polyanion.

The complex DNA/PLL (1:6) + 200  $\mu$ g DS was prepared as in p. 80. 25  $\mu$ g/95  $\mu$ g/200  $\mu$ g in 0.5 ml 25 mM HEPES, pH 8.0. then it was diluted up to 1.5 ml. 0.5 ml of this solution (17  $\mu$ g/ml) was tested for Iso

No.	FI	Conc.
1	173.291	-7534.4 DNA/PLL (1:6)
2	613.903	-26691 — " — +200 $\mu$ g DS
3	219.280	-9533.9 DNA/PLL aft. cent.
4	541.387	-23538 — " — +200 $\mu$ g DS + aft. cent.
5	723.397	-31452 DNA/PLL (FI) in salt.
6	984.784	-42816 — " — +200 $\mu$ g DS in salt.
7	56.981	-2477.4 DNA/PLL in salt aft. cent.
8	588.275	-25577 — " — +200 $\mu$ g DS in salt aft. cent.